METHODS FOR DETECTING AND MONITORING CANCER USING EXTRACELLULAR RNA

This application is a continuation-in-part of U.S. Serial No. 10/013,868, filed October 30, 2001, which is a continuation of U.S. patent application, Ser. No. 09/155,152, filed Sep. 22, 1998, now U.S. Pat. No. 6,329,171 B1, which is a U.S. national phase application filed pursuant to the provisions of 35 U.S.C. §371 of International Application, Serial No. PCT/US97/03479, filed Mar. 14, 1997, which claims the benefit of the filing date of Provisional U.S. patent application, Ser. No. 60/014,730, filed Mar. 26, 1996, the entire disclosure of each of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Ribonucleic acid (RNA) plays an essential role in the translation of the genetic code to produce proteins necessary for cellular function, both in normal cells and neoplastic or diseased cells. In particular, RNA including transfer RNA, messenger RNA or messenger-like RNA, and ribosomal RNA carry and translate the genetic code to sites of protein production. Further, double-stranded RNA plays an important role in silencing genetic expression. Other RNA species are found within ribonucleoproteins. For example, telomerase RNA is a critical component of telomerase, an important ribonucleoprotein highly expressed in most cancers. The pathogenesis and regulation of cancer is thus dependent upon RNA-mediated translation of specific genetic code, often reflecting mutational events or other alterations within deoxyribonucleic acid (DNA), including epigentic alterations such as hypermethylation, microsatellite alterations, loss of heterozygosity, translocations, deletions, and point mutations. Further, other RNA species

and their associated proteins, although not necessarily being directly involved in neoplastic pathogenesis or regulation, may provide recognizable characterization of neoplasia or disease by being inappropriately expressed or elevated. Such overexpression of RNA thus can delineate cancer or other disease. Recognition of the presence or overexpression of specific RNA can enable identification, detection, inference, monitoring, or evaluation of any neoplasm, whether benign, malignant, or premalignant, in humans and animals.

United States Patent No. 6,329,179 B1, incorporated herein in its entirety, teaches that both tumor-associated and non-tumor associated RNA are detectable in plasma and serum. Total RNA, to be understood in cancer patient to comprise both tumor-associated and non-tumor-associated RNA and further being heterogeneous RNA, can be extracted from plasma or serum, the RNA of interest or its cDNA is amplified qualitatively or quantitatively, and the amplified product of an RNA or cDNA species of interest detected. Of particular note, U.S. patent no. 6,329,179 B1 teaches that extracellular RNA is present in greater amount when obtained from cancer patients than from healthy individuals. Subsequent art supports these teachings by demonstrating that extracellular RNA of various RNA species are detectable in bodily fluids, for example in co-owned United States Patent no. 6,607,898; Kopreski *et al.*, 1999, *Clin. Cancer Res.* 5: 1961-1965; Dasi *et al.*, 2001, *Lab. Investigation* 81: 767- 769; Hasselmann *et al.*, 2001, *Oncol. Rep.* 8: 115-118; Ng *et al.*, 2002, *Clin. Chem.* 48: 1212-1217; Chen *et al.*, 2000, *Clin. Cancer Res.* 6: 3823-3826; Silva *et al.*, 2001, *Clin. Cancer Res.* 7: 2821-2825; Silva *et al.*, 2001, *Oncol. Rep.* 8: 693-696; Gal *et al.*, 2001, *Ann. NY Acad. Sci.* 945: 192-194; Durie *et al.*, 2000, *Acta Oncol.* 39: 789-796; Fleischhacker *et al.*, 2001, *Ann. NY Acad. Sci.* 945: 179-188; Miura *et al.*, 2003, *Oncology* 64: 430-434; and Kopreski *et al.*, 2001, *Ann. NY Acad. Sci.* 945: 172-178, said

references incorporated herein in their entirety. Detection of tumor-associated RNA in plasma or serum thus provides a method for detecting, diagnosing, inferring, or monitoring cancer or premalignancy in a human or animal.

Thus, extracellular total RNA is increased in the plasma, serum, or other bodily fluid of humans or animals with cancer and other disease. Thus, there is a need in the art for methods of comparing the amount or concentration of plasma or serum total RNA, including both tumor and non-tumor related RNA, in a subject to that of healthy individuals, to permit diagnosis, detection, inference, or monitoring of diseases such as cancer in a human or animal that are associated with increased extracellular total RNA in said bodily fluids. Further, there is a need for methods comparing the amount or concentration of either total extracellular RNA or non-tumor extracellular RNA species or tumor-related extracellular RNA species from a bodily fluid to that in a healthy individual for diagnosing, detecting, inferring, or monitoring cancer and other neoplastic diseases in a human or animal.

SUMMARY OF THE INVENTION

The invention provides methods for diagnosing, detecting, inferring, evaluating, or monitoring disease, and particularly cancer or other neoplastic disease, in a human or animal, by determining the amount, concentration, or other quantitative or comparative assessment of extracellular total RNA, or of one or more specific RNA species, wherein the RNA species may be either non-tumor related RNA or tumor-related RNA, in a plasma, serum, or bodily fluid specimen from the human or animal. Comparison of the qualitative or quantitative amount or concentration of RNA from said human or animal specimen is

made to a comparative specimen RNA assessment, wherein said assessment may comprise a set of previously determined reference range values, to one or a plurality of any of the following reference groups: a healthy human or animal; a human or animal population without cancer; a human or animal with cancer or neoplastic disease; a human or animal population with cancer; a human or animal population with neoplastic disease; a human or animal population with metastatic or advanced cancer; or a previous specimen from the human or animal under evaluation. If the amount or concentration of total extracellular RNA or a specific extracellular RNA species from the bodily fluid of the human or animal is demonstrated to be greater than the amount or concentration present in a healthy human or animal, more specifically a human or animal without cancer, then disease, and particularly cancer or neoplastic disease, is demonstrated or inferred in the evaluated human or animal. Similarly, if the amount or concentration of total extracellular RNA or a specific extracellular RNA species from the bodily fluid of the human or animal is demonstrated to be in the range of a similar amount or concentration found in the comparative specimen of a human or animal or population with cancer, then cancer or neoplastic disease is established in the evaluated human or animal. The methods provided by the invention comprise qualitative or quantitative determination of the amount or concentration of total extracellular RNA or specific extracellular RNA species in a bodily fluid specimen by any of means known to the art, including but not limited to nucleic acid amplification, signal amplification, spectroscopy including mass spectroscopy, and hybridization methods using detectably-labeled probes.

According to a first aspect of the present invention, there is provided methods for detecting, diagnosing, inferring, or monitoring disease, particularly cancer or neoplastic

disease in a human or animal, the method comprising the steps of extracting total extracellular RNA from plasma or serum or other bodily fluid specimen of the human or animal, determining quantitatively or qualitatively the amount or concentration of total extracellular RNA from a fraction of said specimen, comparing said amount or concentration of extracellular RNA obtained from the fraction of said specimen to the amount or concentration of extracellular RNA in reference group, wherein said comparison thereby detects, diagnoses, infers, or monitors a cancer or neoplastic disease in a human or animal. In preferred embodiments, the reference group is a human or human population of individuals without cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of total extracellular RNA in the fraction of the specimen is greater than the amount or concentration of total extracellular RNA found in the reference group. In alternative preferred embodiments, the reference group is a human or human population of individuals with cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of total extracellular RNA in the fraction of the specimen in not significantly less than the amount or concentration of total extracellular RNA found in the reference group.

According to another aspect of the present invention, there is provided methods for detecting, diagnosing, inferring, or monitoring disease, particularly cancer or neoplastic disease in a human or animal, the method comprising the steps of extracting total extracellular RNA from plasma or serum or other bodily fluid specimen of the human or animal, determining quantitatively or qualitatively the amount or concentration of one or a plurality of extracellular RNA species from a fraction of said specimen, comparing said amount or concentration of one or a plurality of extracellular RNA species obtained from

the fraction of said specimen to the amount or concentration of one or a plurality of corresponding extracellular RNA species in reference group, wherein said comparison thereby detects, diagnoses, infers, or monitors a cancer or neoplastic disease in a human or animal. In preferred embodiments, the reference group is a human or human population of individuals without cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of one or a plurality of extracellular RNA species in the fraction of the specimen is greater than the amount or concentration of one or a plurality of extracellular RNA species found in the reference group. In alternative preferred embodiments, the reference group is a human or human population of individuals with cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of one or a plurality of extracellular RNA species in the fraction of the specimen is not significantly less than the amount or concentration of one or a plurality of extracellular RNA species found in the reference group.

According to another aspect of the present invention, there are provided methods for detecting, diagnosing, inferring, or monitoring cancer or neoplastic disease in a human or animal, the method comprising the steps of obtaining a plasma or serum specimen from the human or animal, determining directly on a portion of said specimen the amount or concentration of total extracellular RNA in a portion of the plasma or serum specimen, comparing said amount or concentration to that of a reference group, wherein said comparison thereby detects, diagnoses, infers, or monitors a cancer or neoplastic disease in a human or animal. In preferred embodiments, the reference group is a human or human population of individuals without cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of total extracellular RNA in the

fraction of the specimen is greater than the amount or concentration of total extracellular RNA found in the reference group. In alternative preferred embodiments, the reference group is a human or human population of individuals with cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of total extracellular RNA in the fraction of the specimen is not significantly less than the amount or concentration of total extracellular RNA found in the reference group.

Alternatively, the invention provides methods for detecting, diagnosing, inferring, or monitoring cancer or neoplastic disease in a human or animal, the method comprising the steps of obtaining a plasma or serum specimen from the human or animal, determining directly on a portion of said specimen the amount or concentration of one or a plurality of extracellular RNA species in a portion of the plasma or serum specimen, comparing said amount or concentration to that of a reference group, wherein said comparison thereby detects, diagnoses, infers, or monitors a cancer or neoplastic disease in a human or animal. In preferred embodiments, the reference group is a human or human population of individuals without cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of one or a plurality of extracellular RNA species is the fraction of the specimen in greater than the amount or concentration of one or a plurality of extracellular RNA species found in the reference group. In alternative preferred embodiments, the reference group is a human or human population of individuals with cancer, and cancer or neoplastic disease is detected, diagnosed or inferred when the amount or concentration of one or a plurality of extracellular RNA species in the fraction of the specimen is not significantly less than the amount or concentration of one or a plurality of extracellular RNA species found in the reference group.

In a preferred embodiment of the inventive methods, the bodily fluid is blood, plasma, serum, urine, effusions including pleural effusions, ascitic fluid, saliva, cerebrospinal fluid, gastrointestinal secretions, bronchial secretions including sputum, cervical secretions, or breast secretions. In a particularly preferred embodiment, the bodily fluid is plasma or serum.

In preferred embodiments of the inventive methods, the amount of total extracellular RNA, or one or a plurality of extracellular RNA species, is determined quantitatively or qualitatively using a method that is nucleic acid amplification, signal amplification, spectroscopy including mass spectroscopy, or hybridization, preferably to a detectably-labeled probe.

In preferred embodiments of the inventive methods, RNA is extracted from blood, plasma, serum, or other bodily fluid using an extraction method that is a gelatin extraction method; a silica, glass bead, or diatom extraction method; guanidinium thiocyanate acid-phenol based extraction methods; guanidinium thiocyanate acid based extraction methods; phenol-chloroform based extraction methods; by centrifugation through a cesium chloride or similar gradient; or using commercially-available RNA extraction methods, most preferably as provided in a kit comprising instructions from the kit manufacturer.

In preferred embodiments of the invention, RNA extracted from plasma, serum, or other bodily fluid is reverse transcribed to cDNA prior to detection or amplification and detection. In these embodiments, the amount or concentration of RNA is determined by qualitative or quantitative analysis of cDNA or amplified cDNA product.

In preferred embodiments of the invention, extracted RNA or the corresponding cDNA is amplified qualitatively or quantitatively to determine the amount or concentration

of a RNA species, using an amplification method that is, *for example*, polymerase chain reaction, or reverse transcriptase polymerase chain reaction; ligase chain reaction; DNA or RNA signal amplification; amplifiable RNA reporter methods; Q-beta replication; transcription-based amplification; isothermal nucleic acid sequence based amplification; self-sustained sequence replication assays; boomerang DNA amplification; strand displacement activation; cycling probe technology; and any combination or variation thereof.

In preferred embodiments of the inventive methods, detection of amplified RNA or cDNA product is performed using a detection method that is, *for example*, gel electrophoresis; enzyme-linked immunosorbent assay (ELISA), including embodiments comprising biotinylated or otherwise modified amplification primers; hybridization using a specific, detectably-labeled probe, *for example*, a fluorescent-, radioisotope-, or chromogenically-labeled probe; Southern blot analysis; Northern blot analysis; electrochemiluminescence; reverse dot blot detection; high-performance liquid chromatography; and variations thereof.

The methods of the invention particularly provide methods for identifying humans at risk for developing a disease, particularly cancer or other neoplastic disease, or who have a malignancy or premalignancy. The methods of the invention thus provide methods for identifying humans having a malignancy such as breast, ovarian, lung, cervical, colorectal, gastric, pancreatic, bladder, endometrial, brain, kidney, or esophageal cancers, leukemias, lymphomas, melanoma, or sarcomas; and premalignancies including but not limited to colorectal adenoma, cervical dysplasia, cervical intraepithelial neoplasia (CIN), bronchial

dysplasia, atypical hyperplasia of the breast, ductal carcinoma in-situ, atypical endometrial hyperplasia, and Barrett's esophagus.

The invention thus permits the presence of cancerous (malignant) or pre-cancerous (premalignant) cells within a human or animal to be detected or inferred by determining an amount or concentration of RNA in the plasma, serum, or other bodily fluid of said human or animal that exceeds the amount or concentration normally present in the plasma, serum, or other bodily fluid of a human or animal without cancer or pre-malignancy.

The invention also permits the existence of a disease within a human or animal to be detected or inferred by determining an amount or concentration of RNA in the plasma, serum, or other bodily fluid of said human or animal that exceeds the amount or concentration normally present in the plasma, serum, or other bodily fluid of a healthy human or animal.

An advantageous application of this invention is to identify humans or animals with disease.

It is a particularly advantageous application of this invention to identify humans or animals having cancer.

Another advantageous application of this invention is to identify humans or animals having risk for developing cancer.

Another advantageous application of this invention is to identify humans or animals having a premalignant disease.

Another advantageous application of this invention is for monitoring cancer, including response to cancer therapies.

Another advantageous application of this invention is selecting humans or animals for cancer therapies, including surgery, biotherapy, hormonal therapy, anti-sense therapy, monoclonal antibody therapy, chemotherapy, vaccines, anti-angiogenic therapy, cryotherapy, and radiation therapy.

Another advantageous application of this invention is to provide a marker as a guide to whether adequate therapeutic effect has been achieved, or whether additional or more advanced therapy is required, and to assess prognosis in a patient.

Another advantageous application of this invention is to provide an indicator of a relapsed cancer following therapy, or impending relapse, or treatment failure.

Another advantageous application of this invention is to identify humans or animals who might benefit from additional diagnostic procedures, wherein said procedures include but are not limited to surgery, biopsy, needle aspiration, radiologic imaging including X-ray, MRI, and CT scanning, radionucleotide imaging, colonoscopy, sigmoidoscopy, bronchoscopy, endoscopy, PET scanning, stool analysis, sputum analysis, cystoscopy, pelvic examination, and physical examination.

The invention also provides diagnostic kits enabling quantitative or qualitative assessment of total RNA or specific RNA species in plasma or serum, wherein a reference range for normal values or cancer values is provided to enable identification or selection of a human or animal with or at risk for cancer.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to methods for detecting, diagnosing, inferring, or monitoring cancer or neoplastic disease in a human or animal by assessing the amount or concentration of RNA, particularly extracellular RNA, in plasma, serum, or other bodily fluid from said human or animal, and comparing the amount or concentration of extracellular RNA obtained from the human or animal with the amount or concentration of RNA found for a reference individual, group or population of known disease status. In particular, the invention provides methods for detecting, inferring or monitoring the presence of cancerous or precancerous cells in a human or animal, whether from a non-hematologic neoplasm (*i.e.*, a solid-tumor) or from a hematologic malignancy (such as leukemia, lymphoma, myeloma, etc.). The methods of the invention determine an amount, concentration or other quantitative or comparative assessment of RNA from a bodily fluid specimen obtained from a human or animal, wherein the RNA can be either total extracellular RNA, or one or a plurality of specific RNA species or multiple specific RNA species. RNA species may be either tumor-related RNA or non-tumor related RNA. Total extracellular RNA will be recognized as comprising both tumor-related and non-tumor-related RNA when obtained from a patient with cancer or other neoplastic disease. In preferred embodiments, the bodily fluid is blood, plasma, serum, urine, effusions including pleural effusions, ascitic fluid, saliva, cerebrospinal fluid, gastrointestinal secretions, bronchial secretions including sputum, cervical secretions, or breast secretions. Plasma and serum are particularly preferred bodily fluids, but any bodily fluid comprising extracellular RNA, and particularly disease-associated extracellular RNA is useful in the practice of the methods of this invention.

As used herein, the terms "disease-associated," "disease-related," "tumor-related" and "non-tumor-related" are intended to encompass particular RNA species, as well as total extracellular RNA. It will be understood that certain RNA species, such as oncogenic RAS, p53, and other RNA species, are recognized in the art as being associated with the existence of cells comprising a disease state, particularly neoplastic disease, malignancy or premalignancy. RNA species are "disease-associated," "disease-related," "tumor-related" when their presence as a component of total extracellular RNA is indicative of the existence of a disease, particularly a neoplastic disease. "Non-tumor-related" RNA species, on the other hand, comprise RNA species component(s) present in healthy individuals; such species may also be present in individuals bearing disease-associated, disease-related, or tumor-related extracellular RNA species as well. It will be recognized that in certain embodiments of the methods of this invention, detecting a lack of expression of an RNA species comprising non-tumor-related RNA may indicate the existence of disease in said human or animal.

As used herein, the term "quantitative" when applied to an amplification method or to detection of total extracellular RNA or one or a plurality of RNA species thereof is intended to indicate that the method or determination performed provides an accurate, reliable and reproducible measure of the amount or concentration of total extracellular RNA or one or a plurality of RNA species thereof, based on a calculated or experimentally-determined measurement of the amount or concentration of total extracellular RNA or one or a plurality of RNA species thereof. In preferred embodiments, the method includes amplification of a standard or control RNA species that is used to determine the amount or concentration of total extracellular RNA or one or a plurality of RNA species thereof.

As used herein, the term "qualitative" when applied to an amplification method or to detection of total extracellular RNA or one or a plurality of RNA species thereof is intended to indicate that the method or determination performed provides a relative measure of the amount or concentration of total extracellular RNA or one or a plurality of RNA species thereof, based on a comparison of the amount or concentration of total extracellular RNA or one or a plurality of RNA species thereof.

Qualitative or quantitative comparison of the amount or concentration of RNA from said human or animal bodily fluid specimen is made in comparison to an RNA specimen or standard from a reference individual, group, or population. Said assessment is made on the basis of a previously-determined reference set of values for said individual, group or population, or alternatively upon a newly determined reference set of values for the individual, group, or population. Comparison to the reference individual, group, or population thereby enables determination of the likelihood that the subject human or animal has a disease, particularly cancer or neoplastic disease such as premalignancy, wherein if the amount or concentration (or similar comparative RNA indicator) of total extracellular RNA or of one or a plurality of specific RNA species thereof from the bodily fluid of the subject human or animal is demonstrated to be greater than the amount or concentration (or similar comparative indicator) present in individuals, groups, or populations without disease, particularly cancer or neoplastic disease, then a disease, particularly cancer or an increased risk of cancer (*for example*, due to the existence of a premalignancy) will be inferred in the human or animal subject. Similarly, if the amount or concentration of total extracellular RNA, or of one or a plurality of specific RNA species thereof in the bodily fluid of the subject is within the range of a group or population with a disease, particularly

cancer or neoplastic disease such as a premalignancy, then a disease, particularly cancer or an increased risk of cancer (*for example*, due to the existence of a premalignancy) will be inferred in the human or animal subject. If the amount or concentration of total extracellular RNA, or of one or a plurality of specific RNA species thereof in the bodily fluid of the subject is less than the range for patients with cancer, or within the range of the healthy population, then the risk of disease, particularly cancer or an increased risk of cancer (*for example*, due to the existence of a premalignancy) will be less. It will be recognized that the limits of the reference range values may be set in a manner that determines a sensitivity or specificity or positive predictive value or negative predictive value for the assay, or otherwise provides the probability of the assay correctly identifying a subject with cancer or neoplasm. Thus, in this manner the reference range for a group or population can be defined that increases the sensitivity or specificity of the assay.

It is to be recognized that a variety of individuals, groups, or populations will provide suitable reference values that enable discrimination of abnormal (disease-, and more particularly cancer-, related) and normal amounts or concentrations of total extracellular RNA, or of one or a plurality of specific RNA species thereof in the bodily fluid of the subject. Appropriate reference individuals, groups, or populations include but is not limited to: a healthy human or animal, more specifically a human or animal population without neoplastic disease (cancer or premalignancy) or a human or animal population without cancer; a human or animal population with a disease, more specifically a human or animal population with neoplastic disease (cancer or premalignancy) or a human or animal population with cancer; a previously-isolated bodily fluid specimen from the human or animal under evaluation corresponding to a known disease or health state. In addition, it

will be recognized that certain defined groups or populations will provide useful reference values to assess probability of disease, particularly cancer or premalignancy, in a subject, including but not limited to: groups and populations defined by gender and the presence or absence of disease, particularly cancer or premalignancy; groups and populations defined by race or ethnicity and presence or absence of disease, particularly cancer or premalignancy; groups and populations defined by non-neoplastic diseases; groups and populations defined by specific tumor types; groups and populations defined by stage or extent of cancer of a particular type; groups and populations defined by certain environmental or occupational risks for cancer, such as smokers or workers occupationally exposed to carcinogens; and groups and populations defined by genetic or family risk for cancer. It is to be understood that the comparative assessment of the subject's total extracellular RNA in a bodily fluid such as blood plasma or serum to reference groups and populations may be made by either non-statistical or statistical analysis, as is known to the art.

In particularly preferred embodiments of the invention, the bodily fluid is blood plasma or serum. Either fresh (*i.e.*, never frozen) blood plasma or serum, or frozen (stored) and subsequently thawed plasma or serum may be used for purposes of these embodiments. In a preferred embodiment the blood is processed soon after drawing, preferably within 24 hours and most preferably within 6 hours, to minimize any degradation of nucleic acids. While early processing is not a requirement of the methods of the invention, it will be recognized that variations of early processing can be employed as set forth below, without limitation implied. In one aspect, the blood may be initially processed to stabilize the RNA or to stabilize phospholipids encapsulating the extracellular RNA, or to inhibit nucleases

present in blood. Stabilizing agents or inhibitors may be provided within kits according to the invention or within venipuncture tubes or devices. Such initial processing is useful if specimen transport or work schedules will result in processing delays. In another aspect, initial processing may be performed by hybridizing the RNA or binding associated apoptotic bodies or other RNA encapsulated particles to solid substrates shortly after venipuncture, preferably using reagents provided in a kit of this invention or as part of specialized blood collection systems. It is preferred that the processing of the specimen from the human or animal subject and from the reference group or population be handled in a similar or like manner to the extent practical, or alternatively the effect due to variations in specimen processing defined and comparisons appropriately adjusted.

In a preferred embodiment, blood is first collected by venipuncture and kept on ice until serum or plasma is separated from whole blood, for example using centrifugation methods gentle enough not to lyse blood cells. While a considerable range of centrifugation speeds may be employed, centrifugation at high speeds (such as beyond 100,000 x g) for prolonged periods should be avoided to prevent clearance of RNA-containing apoptotic bodies or other encapsulated extracellular RNA particles from the supernatant. Non-limiting examples of suitable conditions is centrifuging a blood specimen at a range of 300 to 5,000 x g for five to thirty minutes, or fractionating by other standard methods to produce plasma or serum will suffice. Sera or plasma obtained in this manner can be assayed directly or stored frozen, for example at –20 to –80 degrees centigrade until further analysis according to the methods of this invention.

In a preferred embodiment of the invention, extracellular RNA in plasma or serum or other bodily fluid of the human or animal is assayed by extracting total extracellular

RNA from plasma or serum or other bodily fluid of the human or animal, determining quantitatively or qualitatively the amount or concentration of total extracellular RNA, or one or a plurality of specific RNA species thereof comprising a portion of the total extracellular RNA, and comparing said amount or concentration obtained from the human or animal to the total extracellular RNA, or one or a plurality of specific RNA species thereof from a reference group, wherein said comparison detects, diagnoses, infers, or monitors a disease, particularly cancer or neoplastic disease in the human or animal. Bodily fluids are preferably separated into essentially cellular and non-cellular components, using centrifugation or other fractionation techniques, and total extracellular RNA thereafter extracted from the non-cellular components.

In the practice of the methods of this invention, total extracellular RNA can be extracted from bodily fluid using methods well-known to the art, including but not limited to gelatin extraction method; silica, glass bead, or diatom extraction method; guanidinium thiocyanate acid-phenol based extraction methods; guanidinium thiocyanate acid based extraction methods; centrifugation through a cesium chloride or similar gradient; phenol-chloroform based extraction methods; hybridization and immunobead separation; or commercially available RNA extraction methods. Methods of RNA extraction are further provided in U.S. Patent no. 6,329,179 B1, incorporated herein in its entirety by reference. If plasma or serum had been previously frozen, upon assay it should be thawed rapidly, *for example* in a warm water bath at about 37 degrees centigrade, and thereafter RNA rapidly extracted to minimize degradation thereof.

However, it should be understood that extraction of total extracellular RNA is not a requirement for the practice of the methods of this invention. In some embodiments,

methods such as spectroscopic methods including mass spectroscopy, and cytometry can be used for direct analysis of total extracellular RNA or RNA encapsulated particles within the bodily fluid.

The amount or concentration of total extracellular RNA from the bodily fluid is determined quantitatively or qualitatively using nucleic acid (RNA or cDNA) amplification, signal amplification, spectroscopy including mass spectroscopy, or hybridization to a detectably-labeled probe. In a preferred embodiment, a portion of the extracted total extracellular RNA is amplified or signal amplified qualitatively or quantitatively. Total extracellular RNA extracted from blood plasma or serum or other bodily fluid may first be reverse transcribed to cDNA, whereupon the cDNA is amplified or signal amplified qualitatively or quantitatively. In preferred embodiments, amplification is performed using primers or probes that are specific for particular RNA or cDNA species, wherein the RNA or its cDNA may be a non-tumor related RNA or a tumor-related RNA. Non-tumor RNA include but are not limited to housekeeper gene RNA, and non-limiting examples of non-tumor RNA include RNA encoding all or a portion of c-abl, porpho-bilinogen deaminase (PBDG), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), retinoic acid receptor (RAR), and β-actin. Examples of tumor-related or tumor-associated RNA not intending to be limiting include tyrosinase RNA, keratin RNA species, prostate specific antigen RNA, alpha-fetoprotein RNA, BCR/abl RNA, carcinoembryonic antigen RNA, p97 RNA, p16 RNA, MUC 18 RNA, PML/RAR RNA, CD44 RNA, EWS/FLI-1 RNA, EWS/ERG RNA, AML1/ETO RNA, MAGE RNA species, beta human chorionic gonadotropin RNA, telomerase-associated RNA including TEP1 RNA, human telomerase RNA template (hTR) RNA and telomerase reverse transcriptase protein (hTERT) RNA, bcl-2 RNA, bax RNA,

survivin RNA, COX-2 RNA, P53 RNA, c-myc RNA, her-2/neu RNA, Von Hippel-Lindau gene RNA, retinoblastoma gene RNA, mutated in colon cancer (MCC) gene RNA, adenomatous polyposis coli (APC) gene RNA, deleted in colon cancer (DCC) gene RNA, epidermal growth factor receptor (EGFR) RNA, epidermal growth factor (EGF) RNA, hn RNP-A1 RNA, hn RNP-A2/B1 RNA, hn RNP-K RNA, 5T4 RNA, DNA methyltransferase RNA, matrix metalloproteinase species RNA, mammaglobin RNA, DD3(PCA3) RNA, glutathione S-transferase RNA, MDR-1 RNA, and JC virus RNA. It will be recognized that the above examples are not intended to be limiting, and any non-tumor or tumor-related RNA species or corresponding cDNA may be detected according to the methods of this invention. Further, it will be recognized that various RNA species are well known to the art, and that the scope of the invention is meant to encompass these RNA species without limitation.

Various amplification methods or signal amplification methods are known in the art and can be used in accordance with the methods of this invention. In preferred embodiments of the methods of the invention, quantitative or qualitative amplification is performed using an amplification or signal amplification method such as polymerase chain reaction; reverse transcriptase polymerase chain reaction; ligase chain reaction; DNA or RNA signal amplification; amplifiable RNA reporters; Q-beta replication; transcription-based amplification; isothermal nucleic acid sequence based amplification; self-sustained sequence replication assays; boomerang DNA amplification; strand displacement activation; cycling probe technology; or any combination or variation thereof. In one aspect of this embodiment, quantitative amplification is performed using the Taqman

technology (Perkin Elmer Biosystems), with primers for the target RNA using a dye-labeled internal primer.

In preferred embodiments, following amplification the RNA or cDNA amplified or signal amplified product is detected in a quantitative or qualitative manner by methods known to the art. In preferred embodiments of the inventive methods, detection of amplified RNA or cDNA product is performed using a detection method selected from a group consisting of gel electrophoresis; ELISA detection including modifications, including biotinylated or otherwise modified primers; hybridization using a specific, fluorescent-, radioisotope-, or chromogenically-labeled probe; Southern blot analysis; Northern blot analysis; electrochemiluminescence; reverse dot blot detection; and liquid chromatography, including high-performance liquid chromatography.

Upon amplification and detection of total extracellular RNA or one or a plurality of specific RNA species, most preferably wherein one or a plurality of species of total extracellular RNA is a disease- or tumor-related gene, an amount or concentration or other value allowing comparative assessment is determines, using for example, gel intensity, signal intensity, or color intensity, color, mass, or electrical propensity. Assessment is made to a reference individual, group, or population based upon analysis of said RNA under similar condition and methods, or by extrapolation to similar conditions and methods. If the RNA in the subject specimen is of greater amount, concentration, or other assessment value than that expected for a healthy reference group or population, or within the range for a disease group or population, most preferably a cancer group or population, then disease, most particularly cancer or neoplastic disease, will be thereby diagnosed, detected, inferred, or monitored in the subject human or animal.

In another embodiment of the invention, determination of an amount, concentration, or other comparative assessment is made using total extracellular RNA without amplification prior to detection. For example but not limitation, total extracellular RNA extracted from a bodily fluid may be hybridized and detected without amplification. In this aspect, it is particularly preferred but not required that the extracted RNA be concentrated upon extraction or upon separation from the bodily fluid, using *for example* immunobead capture or hybridization onto a solid substrate, to improve assay sensitivity. In another aspect of this embodiment, extracellular RNA is evaluated by spectroscopy, *for example* by mass spectroscopy or magnetic resonance spectroscopy, or by flow cytometry. In one aspect, fluorometric methods may be employed, *for example* as employed by Kamm and Smith (1972, *Clin. Chem.*18: 519-522), said reference incorporated herein in its entirety.

The methods of the invention identify humans or animals bearing or at risk for developing malignancies including but not limited to tumors of breast, ovarian, lung, cervical, colorectal, gastric, pancreatic, bladder, endometrial, head and neck, brain, kidney, and esophageal tissues, as well as leukemias, lymphomas, melanoma, and sarcomas. The methods of the invention may further be utilized to identify humans or animals with premalignancy, including but not limited to colorectal adenoma, cervical dysplasia, cervical intraepithelial neoplasia (CIN), bronchial dysplasia, bronchial metaplasia, atypical hyperplasia of the breast, ductal carcinoma in-situ of the breast, atypical endometrial hyperplasia, prostatic intraepithelial neoplasia, and Barrett's esophagus. The methods of the invention may be applied to a subject of any age, race, ethnicity or gender, although it is preferred that the reference group or population include individuals of similar age (child, adult, elderly) and sex (male, female).

The invention permits detection, diagnosis, and monitoring of disease, particularly cancer and premalignancy, and identification of individuals at risk for developing disease, particularly cancer or neoplastic disease such as premalignancy, providing considerable clinical utility. The invention provides methods to identify, stratify, or select a human or animal that might benefit from a therapy, or from a further diagnostic test. The invention permits disease such as cancer to be monitored, including response to cancer therapies, by providing a marker to guide whether therapeutic effect has been achieved, or if more therapy is required, and to assess prognosis.

An advantageous application of the methods of this invention is to allow selection of humans or animals for cancer therapies including surgery, biotherapy, hormonal therapy, anti-sense therapy, monoclonal antibody therapy, chemotherapy, vaccines, anti-angiogenic therapy, cryotherapy, and radiation therapy.

Another advantageous application of the methods of this invention is to provide an indicator of a relapsed cancer following therapy, or impending relapse, or treatment failure.

Another advantageous application of the methods of this invention is to identify humans or animals who might benefit from additional diagnostic procedures, wherein said procedures include but are not limited to surgery, biopsy, needle aspiration, radiologic imaging including X-ray, MRI, and CT scanning, radionucleotide imaging, colonoscopy, sigmoidoscopy, bronchoscopy, endoscopy, PET scanning, stool analysis, sputum analysis, cystoscopy, pelvic examination including PAP, and physical examination.

The invention further provides diagnostic and research kits that enable quantitative, qualitative or other comparative assessment of total RNA or of specific RNA species in plasma, serum, or other bodily fluids. In one aspect, a kit according to this aspect of the

invention can provide a reference range for normal values or values that are disease-related under conditions that enable identification or selection of a human or animal with a disease, most particularly cancer or neoplastic disease. In another aspect kits of this invention provide reagents for extracting total extracellular RNA from the bodily fluid, or reagents and/or probes and primers for the amplification of said RNA, or reagents and materials for the detection of RNA product, or reagents for hybridization of RNA, or standards and controls for the analysis of the test, or reagents or devices or tubes for collecting, handling, or storage of the bodily fluid, or any combination or variation thereof, wherein further the reagents may be standardized to be comparable with reagents used to define RNA values for the reference population.

The methods of the invention and preferred uses for the methods of the invention are more fully illustrated in the following Examples. These Examples illustrates certain aspects of the above-described method and advantageous results. These Examples are shown by way of illustration and not by way of limitation.

Example 1

Defining reference values in human populations:

The presence or absence of tyrosinase RNA in serum was evaluated in qualitative fashion for two human populations, one comprising 20 healthy individuals, and the second comprising 6 patients with malignant melanoma. Total RNA was extracted from serum using a commercial kit (Perfect RNA: Total RNA Isolation Kit, 5 Prime – 3 Prime Inc., Boulder, Colorado) according to manufacturer's instructions. The extracted RNA from 50

microliters of serum was then reverse transcribed and tyrosinase cDNA amplified by nested polymerase chain reaction using tyrosinase-specific primers. Amplified product was electrophoresed through a 4% agarose gel in 1 x TBE buffer at 100 volts for 2 hours and stained with ethidium bromide and detected. The amount or concentration of tyrosinase RNA in serum was below limits of detection in all 20 healthy individuals. Conversely, 4 of 6 patients with melanoma had detectable tyrosinase RNA in their serum as determined by detection of a tyrosinase-specific amplified fragment of the predicted size. These results thus define the reference value for extracellular tyrosinase RNA in serum from healthy individuals as being either very low in amount and concentration, or as being qualitatively non-detectable. Individuals screened for cancer, particularly melanoma, which demonstrate high or detectable amounts or concentrations of tyrosinase RNA in their serum are thus identified as either having melanoma, or having a high risk for melanoma.

Example 2

Clinical application

A 52 year-old woman with no symptomatic evidence of disease presents for routine cancer screening. Her physician draws a plasma specimen for assay. Total extracellular RNA is extracted from the patient's plasma, and the extracted extracellular RNA amplified quantitatively using Taqman PCR technology for a housekeeping gene RNA or similar standard RNA such as c-abl RNA. In this case the woman's quantitative levels of c-abl RNA exceeds the normal reference range of c-abl RNA in plasma from healthy patients without cancer. The presence of cancer, or a high risk of developing cancer, is therefore

identified for the woman, and the physician is indicated to perform additional diagnostic testing to further define the extent and nature of the woman's cancer.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.